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Highly Efficient Enzyme Reactors Containing Trypsin and Endoproteinase LysC Immobilized on Porous Polymer Monolith Coupled to MS Suitable for Analysis of Antibodies

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Capillary enzymatic microreactors containing trypsin and endoproteinase LysC immobilized on a porous polymer monolith have been prepared and used for the characterization and identification of proteins such as cytochrome c, bovine serum albumin, and high-molecular weight human immunoglobulin G. The hydrophilicity of diol functionalities originating from the hydrolyzed poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith was not sufficient to avoid adsorption of hydrophobic albumin in a highly aqueous mobile phase. Therefore, this monolith was first hydrophilized via photografting of poly(ethylene glycol) methacrylate followed by photografting of a 4-vinyl-2,2-dimethylazlactone to provide the pore surface with reactive functionalities required for immobilization. This new approach reduced the undesired nonspecific adsorption of proteins and peptides and facilitated control of both the enzyme immobilization and protein digestion processes. The enzymatic reactors were coupled off-line with MALDI/TOF MS and/or on-line with ESI/TOF MS. Experimental conditions for digestion were optimized using cytochrome c and bovine serum albumin as model proteins. The optimized reactors were then integrated into a multidimensional system comprised of a monolithic capillary enzyme reactor, an in-line nanoLC separation of peptides using a poly(lauryl methacrylate-*co*-ethylene dimethacrylate) monolithic column, and ESI/TOF MS. With the use of this system, immunoglobulin G was digested at room temperature in 6 min to an extent similar to that achieved with soluble enzyme at 37 °C after 24 h.

For many decades, the digestion of proteins has been used to determine their structure. The bottom-up approach to protein analysis involves digestion, the separation of resulting peptides, and their mass spectrometric detection.[1] This approach is also an integral part of the quality control (QC) and quality assurance (QA) of biotechnology products. The standard protocols include proteolysis in a homogeneous solution containing both proteolytic enzyme and the protein of interest. This approach has significant limitations including slow digestion times, larger volume requirements, and complex sample handling. The proteolysis is typically carried out with only a small amount of the soluble enzyme to avoid the undesired autodigestion. Therefore, the digestion requires several hours to proceed. The most common enzyme currently used in protein characterization is trypsin. Recently, endoproteinases such as GluC, ArgC, and LysC have been gaining more popularity due to their highly specific points of cleavages. They offer additional benefits such as digestion resulting in a smaller number of larger, information-rich peptide fragments that simplifies their separation and facilitates protein identification. These enzymes are also useful in mapping of post-translational modifications such as methionine oxidation since the large peptides allow lower quantization limits to be achieved using typical UV detection. The larger peptides formed during LysC digestion are also more likely to contain multiple sites allowing charging such as internal arginines, which form ions better suited for MS/MS analysis.[2] Endoproteinase LysC is also a more robust enzyme that, compared to trypsin, maintains its activity even at relatively high concentrations of denaturants.

Some of the problems typical of protein digestion using proteolytic enzymes in solution can be precluded with their

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immobilization on a solid support.[3,4] This approach enables a high localized concentration of the enzyme and a significant acceleration of the digestion without any appreciable autodigestion due to the site isolation effect.[5−7] Both particles and monolithic supports have already been used for immobilization and fabrication of highly active enzyme reactors.[3,8−33] In an extensive review, numerous studies related to the use of monolithic supports for immobilization of proteolytic enzymes have been recently summarized.[7] However, most of these studies concerns digestion of low-molecular weight substrates, peptides, and proteins such as myoglobin, cytochrome c, α-lactalbumin, bovine serum albumin, and holo-transferrin, which have molecular masses that do not exceed 80 000 Da. In contrast, only one report concerns digestion of a large protein, human immunoglobulin G, using papain immobilized on a large poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith modified with 1,6-diaminohexane and glutaraldehyde followed by an off-line separation of the peptides in SEC mode.[17] However no report could be found demonstrating a system involving on-line coupling of an immobilized enzyme reactor prepared from a proteolytic enzyme immobilized on a porous polymer monolith in a capillary with a capillary separation column operating in the reversed-phase mode coupled to an ESI-TOF mass spectrometer and use of this system for analysis of IgG.

The size of samples in some proteomic studies is very small and may be represented by only a few cells. Therefore, miniature immobilized enzyme reactors placed in capillaries or microfluidic devices are highly desirable in proteomic studies. Additional practical benefits of the immobilized enzyme reactor are the possibility for direct coupling with liquid chromatography and mass spectrometry,[20,24,29] which eliminates possible sample loss or contamination during manual handling. Also, the immobilized enzyme reactor can be reused many times, thus saving costs.

In the present work, we demonstrate a novel approach to modification of the surface chemistry of monolithic poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) via multistep/multilayer photografting to obtain support for enzyme immobilization with both reactive azlactone functionalities and largely eliminated nonspecific adsorption of proteins and peptides. This support placed in a 100 μm i.d. fused silica capillary is then used for covalent immobilization of trypsin and endoproteinase LysC. Operational parameters of the reactor were optimized using the digestion of model proteins cytochrome c and BSA. The digestion was monitored by both MALDI-TOF MS and ESI-TOF MS. The optimized reactor was then integrated into a system also comprised of an on-line nanoLC column for the separation of the peptides and ESI-TOF MS detection. This system was then adjusted to facilitate analysis of high-molecular weight human IgG.

EXPERIMENTAL SECTION

Materials and Methods. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (TPCK) treated trypsin from bovine pancreas, endoproteinase LysC from *Lysobacter enzymogenes*, bovine cytochrome c, bovine serum albumin (BSA), fluorescein-labeled BSA, and polyclonal human immunoglobulin G (hIgG) were obtained from Sigma-Aldrich Co. (St. Louis, MO). A nanoACQUITY UPLC system (Waters, Milford, MA) was used for the separations of protein digests. A Micromass LCT time-of-flight mass spectrometer (Manchester, U.K.) equipped with a PicoView nanospray source (New Objective, Woburn, MA) was used in all experiments, and the ESI-TOF measurements were carried out in positive ion mode with a scan range of 400−2500 m/z. MALDI measurements were carried out on a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, MA). Samples were applied using the "dried-droplet" technique and α-cyano-4-hydroxycinnamic acid (CHCA) as a matrix. The 5 mg/mL CHCA solution was prepared in 0.1% trifluoroacetic acid solution in 1:1 acetonitrile. The mass spectrometric measurements were carried out in the positive ion reflectron mode with a scan range adjusted to 500−5000 m/z. Protein identification based on the mass of detected ions was obtained using the MS-Fit peptide mass fingerprinting tool of the Protein Prospector protein digestion database (http://prospector.ucsf.edu).

Modification of the inner walls of the capillaries, preparation of both the monolithic poly(LMA-*co*-EDMA) separation column and the poly(GMA-*co*-EDMA) support as well as immobilization of trypsin on this support are detailed in the Supporting Information. A scheme depicting the preparation of the optimized support based on poly(GMA-*co*-EDMA) monolith photografted first with ethoxylated hydroxyethyl methacrylate and then with 2-vinyl-4,4-dimethylazlactone is shown in Figure 1 and detailed in the experimental procedure described in the Supporting Information. The extent of protein adsorption on the unmodified and the

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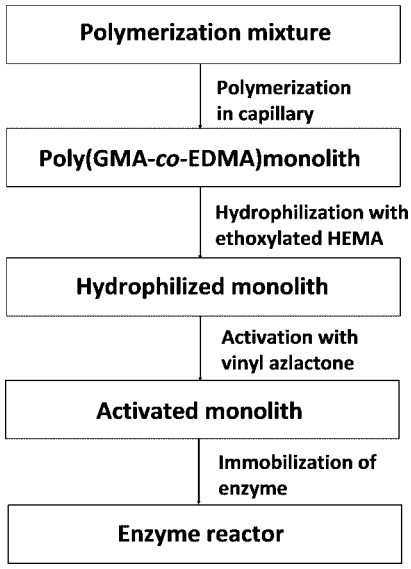

Figure 1. Schematic of the preparation of the optimized monolithic support for the immobilization of proteolytic enzymes.

PEGMA-photografted poly(GMA-*co*-EDMA) monolithic column was evaluated using a fluorescence assay developed previously[34] and is also presented in the Supporting Information.

Immobilization of Trypsin and Endoproteinase LysC on Grafted Support. Trypsin was dissolved (2.5 mg/mL) in 50 mmol/L phosphate buffer, pH 7.2, containing 0.25 mg/mL benzamidine. The enzyme solution was pumped through the monolith at a flow rate of 0.25 μL/min for 3 h.

Endoproteinase LysC was dissolved (0.5 mg/mL) in 250 mmol/L Tricine, 50 mmol/L EDTA, pH 8.0, containing 0.05 mg/mL benzamidine. The enzyme solution was pumped through the monolith at a flow rate of 0.15 μL/min for 1.5 h.

After the immobilization reaction, the monoliths were rinsed with 1 mol/L ethanolamine in 50 mmol/L phosphate buffer, pH 7.2, at a flow rate of 0.15 μL/min for 1 h to quench all unreacted azlactone functionalities. Finally, the monolithic reactor was washed with 50 mmol/L ammonium acetate solution, pH 6.7, at a flow rate of 0.5 μL/min and stored in the same solution at 4 °C.

Sample Preparation for Protein Digestion. Protein (BSA, hIgG) was dissolved in 100 mmol/L ammonium bicarbonate, pH 8.2, containing 8 mol/L urea to obtain a solution with a concentration of 5 mg/mL and reduced after admixing 100 μL of 45 mmol/L dithiotreitol at 60 °C for 40 min. After the solution was cooled to room temperature, the proteins were alkylated with 100 μL of 100 mmol/L iodoacetamide for 30 min at room temperature. Cytochrome c without any previous treatment was dissolved in 100 mmol/L ammonium bicarbonate solution, pH 8.2, to obtain a solution with a concentration of 1 mg/mL. Before the digestion, salts were removed from denatured protein solutions using C4 ZipTip (Millipore, Billerica, MA). The proteins were eluted with acetonitrile and dried. For digestion, the proteins were dissolved in solution consisting of 50 mmol/L ammonium acetate, pH 8.75, containing 5 or 20% acetonitrile.

Digestion Using Immobilized Enzymes. The digestions were carried out for times varying from 1.5 to 10 min and at temperatures 25−45 °C. The temperature was controlled using a capillary heater (AgilleSLEEVE Analytical Sales and Services, Inc., Pompton Plains, NJ). For on-line digestion, the proteins were pumped through the reactor that was connected to a liquid junction interface for the nanospray source of the spectrometer. Alternatively, the reactor could be connected to a six-port Cheminert switching valve equipped with an electric actuator and a 500 nL injection loop (Vici Valco, Houston, TX). The protein solution was continuously pumped through the reactor and loop using a syringe pump. The valve was switched at specified times with the contents of the loop transferred to the separation column and analyzed. This setup shown in Figure 2 enabled testing digestion of proteins that differ widely in molecular sizes, cytochrome c (MW 11 572), BSA (MW 66 430), and hIgG (MW ~150 000), and separation of peptides to occur independently. A monolithic poly(LMA-*co*-EDMA) column (100 μm i.d.)[29,35] was used for the separations of peptides in protein digests using a gradient of acetonitrile in aqueous 0.1% formic acid at a flow rate of 0.5 μL/min. The peptide fragments from off-line digestions were collected in microvials and analyzed using MALDI-TOF MS, while ESI-TOF-MS was used for on-line experiments.

Digestion Using Soluble Enzyme. Denatured BSA and hIgG were diluted with water to achieve the final urea concentration of 2 mol/L and a protein concentration of 1.25 mg/mL. Cytochrome c was diluted with the digestion buffer and used without any pretreatment. Trypsin or endoproteinase LysC, respectively, was added at a substrate-to-enzyme ratio of 50:1 (w/w) and the solution incubated at 37 °C for 24 h. The proteolysis was terminated by a pH decrease after addition of acetic acid to the solution. The digests were then desalted using a C18 ZipTip following the protocol suggested by the manufacturer. The peptide solutions in microvials were vacuum-dried to pellets that were redissolved in a 50 mmol/L aqueous ammonium acetate solution at pH 8.75 containing 5 and 20% acetonitrile, respectively, to achieve a concentration of 0.3 mg/mL. This was analyzed using MALDI-TOF MS or nanoLC−ESI-TOF MS.

RESULTS AND DISCUSSION

Trypsin Immobilized via Epoxide Functionalities. Use of epoxide groups of glycidyl methacrylate copolymers for immobilization of enzymes has been known for quite some time.[3] Porous polymer monoliths containing this functionality or groups derived from it have also been successfully used for the immobilization of proteolytic enzymes.[10,17,19,21,36,37] It has been believed that pore surface of porous glycidyl methacrylate copolymers after reaction with nucleophiles such as amines includes hydrophilic moieties that minimize the nonspecific adsorption of proteins and facilitate repeated use of the reactor. To evaluate the extent of protein adsorption, we developed simple qualitative fluorescence assay that measured adsorption of highly hydrophobic BSA. A 0.5 mg/mL solution of BSA labeled with fluorescein isothiocyanate was pumped through the monolithic column for 30 min. The column was then washed, and the intensity of fluorescence was observed using an optical microscope. The desired low level of protein adsorption was indicated by a low fluorescence intensity.

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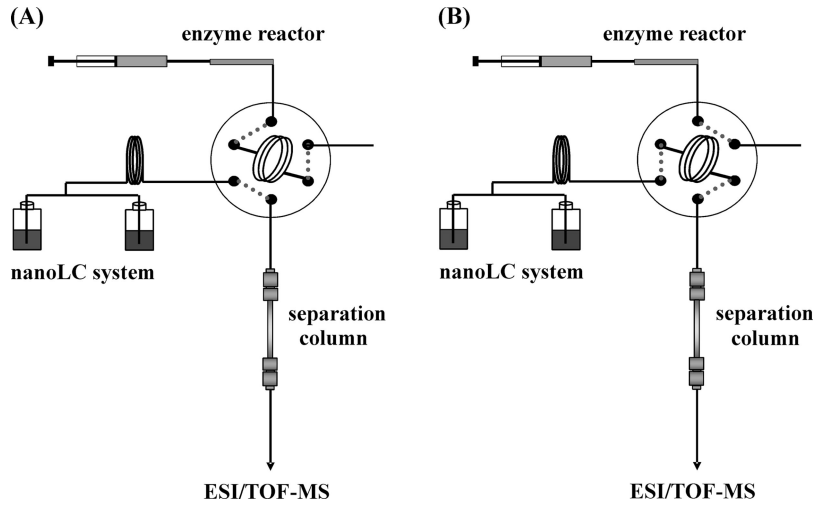

Figure 2. Experimental setup for integration of the enzyme reactor into the nanoLC−ESI-MS system.

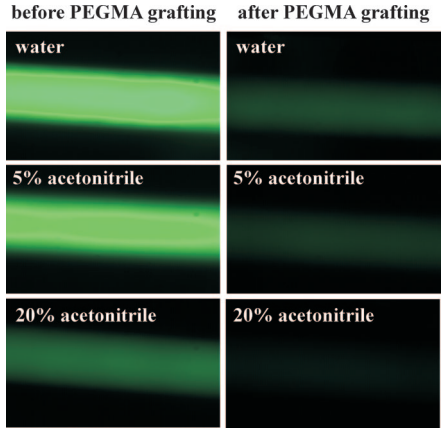

Figure 3. Assay of the sorption of fluorescently labeled BSA on poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith with hydrolyzed epoxy functionalities and after its grafting with PEGMA using a mobile phase containing different percentages of acetonitrile.

Table 1. Sequence Coverage Identification of BSA Using Immobilized and Soluble Trypsin

	sequence coverage, %	
setup	immobilized trypsin 22 °C, 4.5 min	soluble trypsin 37 °C, 24 h
nanoLC−ESI/TOF-MS	80	70
MALDI/TOF-MS	44	64

First epoxide groups of monolithic poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) capillary columns were hydrolyzed using a 0.5 mol/L aqueous sulfuric acid solution[38] to form diol functionalities. These columns were then exposed to the labeled BSA followed by extensive washing with aqueous solutions containing up to 50% acetonitrile. Figure 3 shows that the diol monolith adsorbs a significant amount of protein. At least 20% acetonitrile is required to limit the adsorption to an acceptable level. This assay demonstrated poor performance of the immobilized trypsin reactor based on epoxide functionalities and demonstrates the necessity of using 20% acetonitrile containing digestion buffer to avoid the unwanted nonspecific protein and peptide adsorption on the methacrylate monolith.

The simplest immobilization protocol consists of the direct reaction of amine functionalities of trypsin with epoxide groups of the glycidyl methacrylate monomer units of the monolith. While this reaction is slow at neutral pH, it is much faster at pH above 9.[21] Therefore, we carried out the immobilization of trypsin at pH 10.5 for 5 h. A competitive inhibitor benzamidine was added to the enzyme solution to avoid autodigestion during the reaction. The residual epoxide functionalities were quenched using ethanolamine after the immobilization was completed.

The conditions we used for the digestion of proteins have been previously described.[21] The optimum pH for digestion with immobilized trypsin is in a pH range 8−10 and comparable with that found for its soluble counterpart. Therefore, 50 mmol/L ammonium acetate solution in 20:80 acetonitrile−water, pH 8.75, was used in all our experiments. Under these conditions, a complete digestion of native cytochrome c was achieved in 4.5 min. The residence time of the digested protein in the reactor was controlled by the flow rate. Using shorter digestion times decreased the extent of digestion, and the presence of intact cytochrome was observed in the mass spectrum. The 19.5 cm long immobilized trypsin reactor was then integrated into the nanoLC−MS system. Figure 4 shows chromatograms of cytochrome c digest prepared using both an 18 h digestion with soluble trypsin and a 6 min digestion with immobilized trypsin. The sequence coverage of 93−95% was similar in both approaches. However, it was achieved much faster with the immobilized trypsin.

Although the fast and efficient digestion of a small protein cytochrome c could be obtained, the reactor did not digest large molecular weight proteins such as BSA and hIgG. Changes in digestion time, buffer composition, and the protein concentration did not improve the digestion. The poor performance of the reactor with these large proteins may result from a slow reaction rate of immobilization via the epoxide group and consequently low density of immobilized trypsin. As demonstrated above, the buffer used in the digestion had to contain 20% of acetonitrile to prevent the nonspecific adsorption. Although this solvent does not completely inactivate trypsin,[15,18,39] it likely affects its digestion

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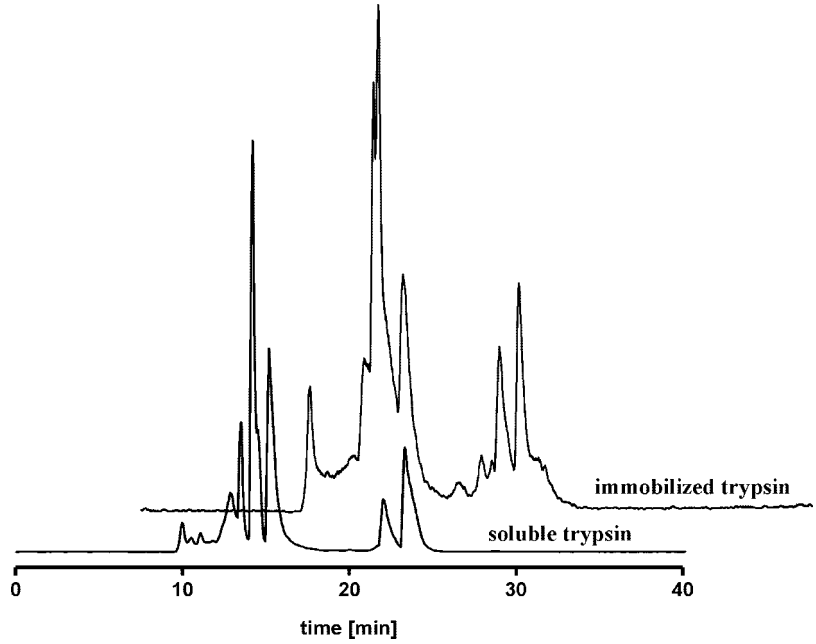

Figure 4. Separation of peptides resulting from the cytochrome c digestion using the trypsin reactor based on epoxide functionalities and soluble trypsin. Digestion conditions: bovine cytochrome c (0.15 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 20% acetonitrile. Immobilized trypsin: 19.5 cm × 100 μm i.d. immobilized trypsin reactor; temperature, 22 °C; digestion time, 6 min. Soluble trypsin: enzyme/protein ratio, 1:50 (w/w); temperature, 37 °C; digestion time, 24 h. Separation conditions: 20 cm × 100 μm i.d. monolithic column; injection time, 5 min; injection volume, 500 nL. Mobile phases: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile. Gradient, hold 20% B for 5 min and then ramp from 20% B to 80% B in 30 min; flow rate, 500 nL/min.

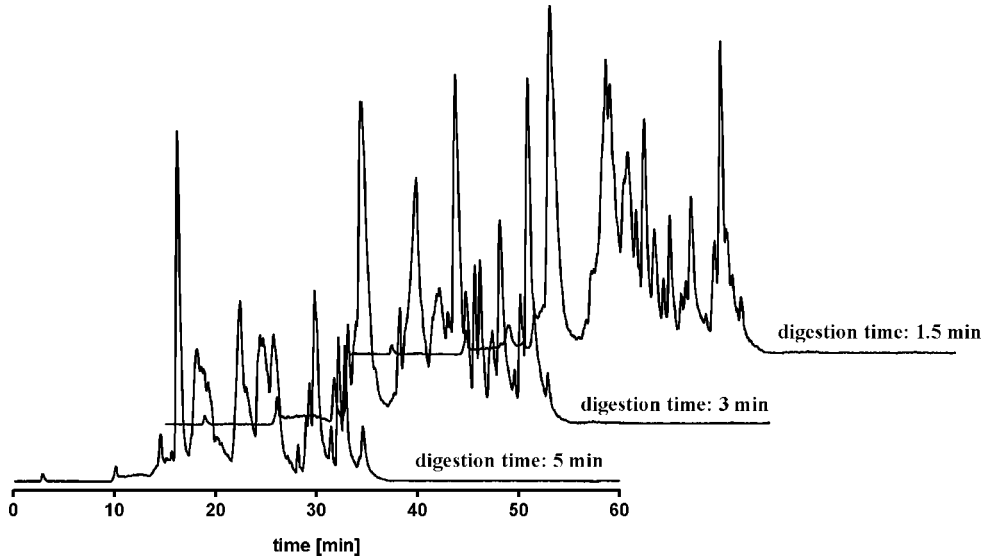

Figure 5. Separation of peptides resulting from the cytochrome c digestion using trypsin reactor based on azlactone functionalities. Digestion conditions: bovine cytochrome c (0.15 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 5% acetonitrile; 17 cm × 100 μm i.d. immobilized trypsin reactor; temperature, 22 °C; digestion time, 1.5−5.0 min. Separation conditions: 20 cm × 100 μm i.d. monolithic column; injection time, 5 min; injection volume, 500 nL. Mobile phases: A, 0.1% formic acid in water; B, 0.1% formic acid in acetonitrile. Gradient, hold 5% B for 5 min, then ramp from 5% B to 80% B in 40 min; flow rate, 500 nL/min.

ability and may be another reason for the failed digestion of BSA and hIgG.

Decrease in Protein Adsorption. Since the monoliths used in the first series of experiments exhibited significant hydrophobic adsorption, the surface chemistry required modification to make the support more hydrophilic and suitable for the preparation of an immobilized trypsin reactor useful in the digestion of higher-molecular weight proteins such as BSA and immunoglobulins.

We have recently developed a simple photografting technique that enables coating of the pore surface of the monoliths with a covalently attached layer of hydrophilic polymers.[34,40] We used the two step process to photograft ethoxylated 2-hydroxyethyl methacrylate, PEGMA, $n = 11$. The epoxide functionalities of generic poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) monolith were first hydrolyzed by sulfuric acid to both quench

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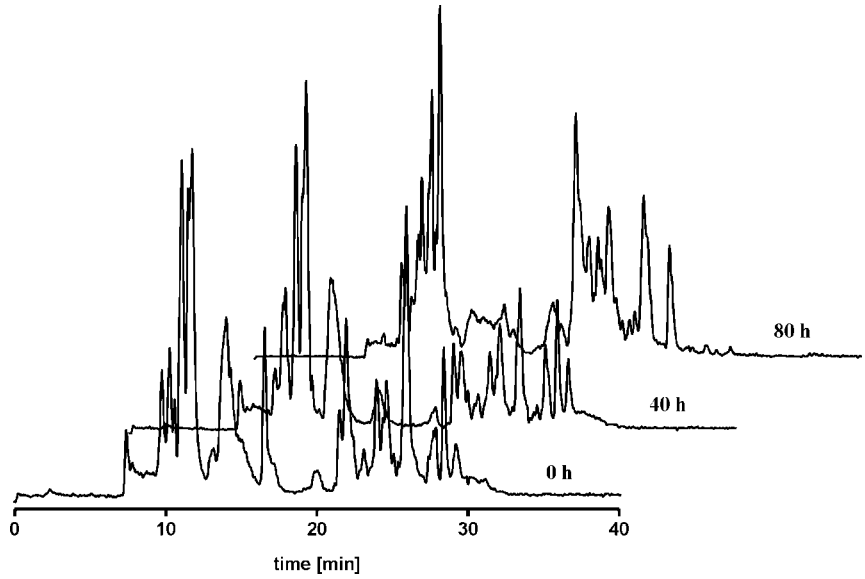

Figure 6. Operational stability of the immobilized trypsin reactor characterized using BSA. Digestion conditions: BSA (0.3 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 5% acetonitrile; 24 cm × 100 μm i.d. immobilized trypsin reactor; temperature, 22 °C; digestion time, 4.5 min. For separation conditions see Figure 5.

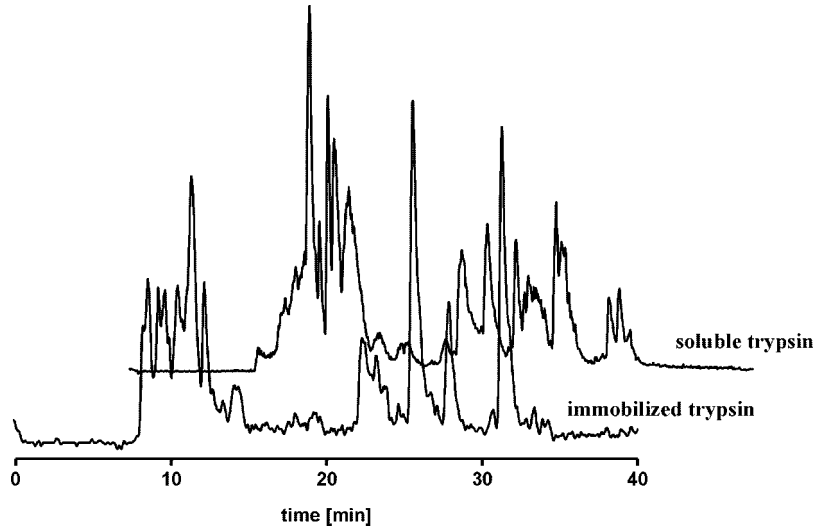

Figure 7. Separation of peptides resulting from hIgG digestion using soluble and immobilized trypsin. Digestion conditions: hIgG (0.5 mg/mL) in 50 mmol/L ammonium acetate pH 8.75 containing 5% acetonitrile. Immobilized trypsin: 12 cm × 100 μm i.d. immobilized trypsin reactor; temperature, 22 °C; digestion time, 4 min. Soluble trypsin: enzyme/protein ratio, 1:50 (w/w); temperature, 37 °C; digestion time, 24 h. For separation conditions see Figure 5.

Table 2. Comparison of Sequence Coverage Identification of Polyclonal hIgG Using Soluble and Immobilized Trypsin

	sequence coverage, %	
chain (C-region)	immobilized trypsin 22 °C, 4 min	soluble trypsin 37 °C, 24 h
Ig γ-1	45.8	50.9
Ig γ-2	36.5	44.5
Ig γ-3	41.7	39.3
Ig γ-4	34.6	34.3
Ig κ	61.3	80.2
Ig λ	80.0	65.7

the epoxide groups and generate diols. In the first step of the two-step photografting procedure, the photoinitiator benzophenone was photografted to the surface for 4 min. This approach is different compared to single step photografting in which both the initiator and the monolith are grafted simultaneously. This allows the use of an organic solvent to dissolve benzophenone to a concentration required to obtain high surface coverage while avoiding polymerization in solution. An aqueous solution of the PEGMA monomer was then photografted in the second step for another 4 min to enhance the hydrophilicity.

The fluorescence assay was used again to characterize the efficiency of the grafting method. Figure 3 indicates that a 5% aqueous acetonitrile was sufficient to reduce BSA adsorption to a tolerable extent. This solvent was also more enzyme friendly and less likely to reduce the activity of the immobilized trypsin. In addition, the lower acetonitrile concentration also benefits the next step, the gradient separation of peptides in reversed-phase chromatography. Since the peptides are focused at the top of the

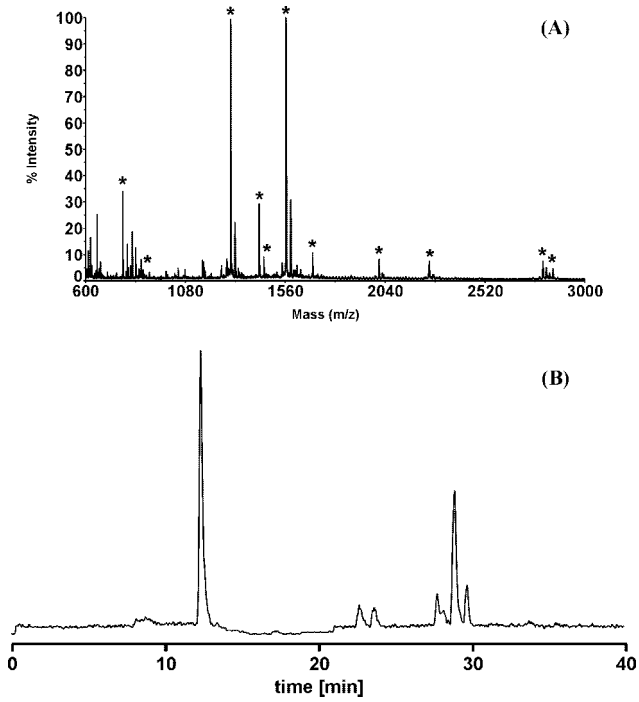

Figure 8. Analyses of peptides resulting from bovine cytochrome c digestion using immobilized endoproteinase LysC. Digestion conditions: bovine cytochrome c (0.15 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 5% acetonitrile; 13.5 cm × 100 μm i.d. reactor with immobilized endoproteinase LysC; temperature, 22 °C; digestion time, 4.0 min. (A) MALDI-TOF MS analysis; (B) nanoLC–ESI-TOF MS analysis. For separation conditions see Figure 5.

lauryl methacrylate based monolithic column, use of highly aqueous solvent facilitates retention of less hydrophobic peptides that were not retained in previously used 20% acetonitrile/water. As a result, a better sequence coverage was obtained since even more hydrophilic peptides are accounted for.

Immobilization Using Azlactone Functionalities. After the hydrolysis of epoxide functionalities and surface grafting of PEGMA, the monoliths do not contain any reactive functionalities that would enable immobilization of enzymes. Therefore, reactivation of the pore surface is necessary. On the basis of good results from our previous research, we grafted vinylazlactone (VAL). The azlactone functionality reacts readily with the amino groups of an enzyme, thus bonding it covalently to the monolithic support. The surface coverage of the azlactone groups is controlled via the concentration of reactive monomer in the photografting mixture and/or grafting time. The concentration of VAL in the mixture was varied in a range of 5–30 wt % at a constant exposure time of 2 min. No appreciable enzyme activity was observed for the BSA digestion using enzyme reactors prepared from a monolith grafted with solutions containing 5 and 10 wt % VAL whereas at a concentration of 25 wt % VAL in the monolithic support was clogged and not permeable. The best enzyme activity without clogging the monolith was obtained with 15 wt % VAL in the photografting mixture.

The first demonstration of proteolytic activity for the immobilized enzyme reactor was carried out using a solution of cytochrome c at different flow rates. Figure 5 confirms that the digestion was very fast since no significant difference was observed between mass spectra obtained after 1.5–5.0 min. Clearly, a complete digestion of cytochrome c can be achieved in 1.5 min although it is likely that a similar extent of digestion could be obtained even in shorter periods of time. Our experimental setup did not however allow use of higher flow rates due to pressure limitations of the syringe pump.

Any specific treatment prior to digestion was not necessary for small proteins such as cytochrome c. In contrast, larger proteins such as BSA or immunoglobulins have to be denatured by urea, their disulfide bridge reduced using dithiotreitol (DTT), and thiol group subsequently alkylated with iodoacetamide (IAA) to enhance exposure of sites of the protein previously inaccessible for proteolysis. The salts present in the protein solution after this treatment have to be removed using the C4 ZipTip to enable the protein to be ready for digestion in the immobilized trypsin reactor followed by MS detection. The effect of denaturing BSA on the extent of digestion and sequence coverage are shown to be significant for both soluble and immobilized trypsin in Table 1. The sequence coverage for treated BSA is almost doubled no matter which enzyme and mass spectrometer was used for digestion and detection. It is worth noting that digestion with trypsin in solution required a temperature of 37 °C and 24 h while a time of only 4.5 min at room temperature was sufficient for the immobilized enzyme reactor.

Operational and Storage Stability. Several identical immobilized trypsin reactors were prepared and tested for the operational and storage stability using the digestion of denatured BSA at a constant reaction time of 4.5 min and a temperature of 22 °C. All reactors maintained their activity for at least 6 months while kept in a refrigerator demonstrating excellent storage stability. One of the reactors was used for 6 h a day repeatedly over a 3 week period to assess the operational stability. This immobilized trypsin reactor maintained its activity for at least 80 h as shown in Figure 6. Only a slight decrease from 80 to 67% in sequence coverage for BSA was observed during this time period.

Digestion hIgG Using Immobilized Trypsin Reactor. Antibodies are one of the most useful tools in clinical immunological and biochemical laboratories. Immobilized antibodies are also utilized to purify substances with affinity chromatography.[41,42] Antibody-based therapies using both polyclonal and monoclonal antibodies are emerging as a powerful therapeutic approach enabling treatment of a variety of diseases including cancer, HIV, immune, infectious, and inflammatory diseases.[43] However, extensive, complex, and slow analytical processes including digestion in solution are required to generate well-characterized biologics. Since quality control in the production process cannot be a bottleneck, a rapid digestion is required. To demonstrate applicability of our immobilized trypsin reactor for the digestion of high-molecular weight proteins, human polyclonal IgG was used as a model. This protein contains four types of heavy chains (γ-1, γ-2, γ-3, and γ-4) and two types of light chains (κ and λ). This immunoglobulin was denatured and treated with DTT and IAA and desalted prior to digestion/nanoLC–MS analysis. Simultaneous experiments were also carried out digesting hIgG with trypsin in solution. The digestion/nanoLC–MS results are shown in Figure 7. The identification of IgG chains based on the known sequences of the constant region summarized in Table 2 resulted in similar values for the digestion using both an immobilized enzyme reactor and soluble trypsin despite the very short digestion time of 4 min and a lower temperature of 22 °C. This

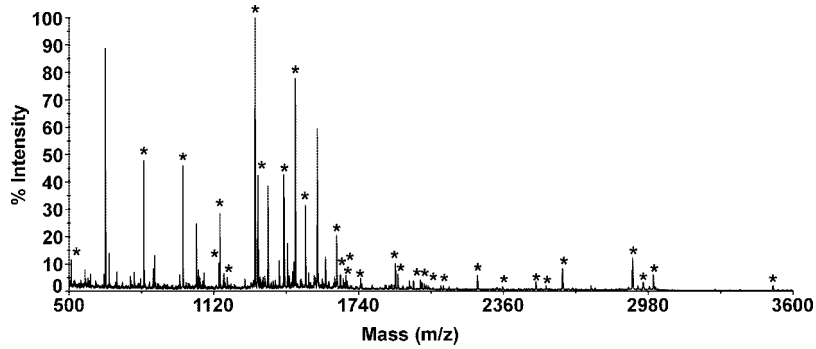

Figure 9. MALDI-TOF MS spectrum of the BSA digest obtained using a reactor with immobilized endoproteinase LysC. Digestion conditions: BSA (0.3 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 5% acetonitrile; 13.5 cm × 100 μm i.d. reactor with immobilized endoproteinase LysC; temperature, 22 °C; digestion time, 6.2 min.

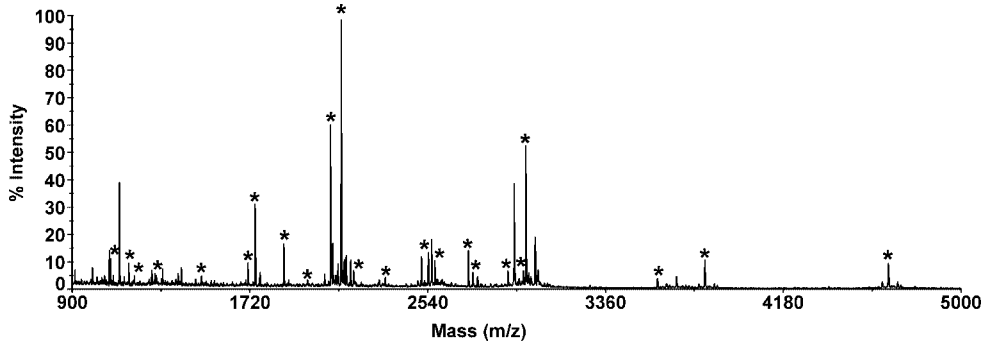

Figure 10. MALDI-TOF MS spectrum of hIgG digest obtained using the reactor with immobilized endoproteinase LysC. Digestion conditions: hIgG (0.5 mg/mL) in 50 mmol/L ammonium acetate, pH 8.75, containing 5% acetonitrile; 13.5 cm × 100 μm i.d. reactor with immobilized endoproteinase LysC; temperature, 37 °C; digestion time, 6.2 min.

Table 3. Sequence Coverage of Polyclonal hIgG Obtained by Using Soluble and Immobilized Endoproteinase LysC and MALDI-TOF MS Detection of Peptides

	sequence coverage, %	
chain (C region)	immobilized LysC 22 °C, 6.2 min	soluble LysC 37 °C, 24 h
Ig γ-1	23.9	48.5
Ig γ -2	20.2	32.5
Ig γ -3	15.2	29.3
Ig γ -4		
Ig κ	29.2	63.2
Ig λ	57.1	

experiment demonstrates that the immobilized enzyme reactor can be used for fast and efficient digestion of a high-molecular weight protein.

Endoproteinase LysC Reactor. Endoproteinases are enzymes enabling cleavage at very specific sites of the protein chain. For example, LysC hydrolyzes peptide bonds at the carboxyl side of lysyl residues. These enzymes are expensive and their immobilization enables repeated use, thus decreasing the price per digest. We have immobilized LysC using support and conditions optimized for trypsin with the exception of a lower concentration of the enzyme in the solution used for immobilization.

Once again, the performance of the immobilized LysC reactor was first tested using cytochrome c in the off-line mode in combination with MALDI-TOF MS. A mass spectrum of a LysC digest obtained after 4 min of residence time is shown in Figure 8A from which a sequence coverage of 73.1% can be calculated. Digestion of cytochrome c in the integrated nanoLC−ESI-TOF MS system is illustrated in Figure 8B affording a sequence coverage 69.2%. Since endoproteinase LysC contains a smaller content of lysine residues required for immobilization, it is likely that the extent of LysC immobilization differs from that of trypsin. This may affect the overall activity resulting in a lower sequence coverage being observed.

The immobilized LysC reactor was also used for the digestion of both denatured BSA and polyclonal hIgG. The digestion time was 6.2 min at a temperature of 22 °C. The mass spectra of the digests of BSA and hIgG are shown in Figures 9 and 10. The sequence coverage of BSA digest obtained using the microreactor was 66.2% as determined by MALDI. This result compares favorably with 55% achieved at 37 °C and 24 h with the enzyme in solution. Results characterizing digestion of hIgG obtained using both MALDI and ESI-MS are summarized in Table 3.

CONCLUSIONS

The porous polymer monoliths can be easily modified via two step photografting including first a highly hydrophilic monomer followed by a reactive monomer. This process forms a double layered structure on the pore surface and affords an excellent support for the immobilization of proteolytic enzymes that exhibit high activity for digestion of not only small proteins but more importantly, also for high-molecular weight proteins such as immunoglobulins G with a molecular mass exceeding 150 000 Da. Direct comparison of the digestions achieved with our immobilized enzyme reactor and with the enzyme in

solution clearly demonstrate advantages of the former in terms of a much shorter reaction time, a lower reaction temperature, and in some cases even a better peptide coverage. Indeed, it might be possible to use small substrate such as benzoylarginine ethyl ester to obtain kinetic parameters of the enzymes in both solution and the immobilized form and compared the results. However, these characteristics would not tell too much about the performance of the immobilized reactors in a real application. Although this report demonstrates only immobilization of trypsin and endoproteinase LysC, it is likely that immobilization of other enzymes using these supports will also be feasible. For example, preliminary experiments with immobilized PNGase F that will be reported elsewhere appear very promising.

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